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Mouse mammary tumor viruses expressed by RIII/Sa mice with a high incidence of mammary tumors interact with the Vβ-2- and Vβ-8-specific T cells during viral infection

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Abstract

The mouse mammary tumor viruses (MMTVs) that induce mammary adenocarcinomas in mice are transmitted from mother to offspring through milk. MMTV infection results in the deletion of specific T cells as a consequence of interaction between the MMTV-encoded superantigen (Sag) and specific Vβ chains of the T cell receptor. The specificity and kinetics of T cell deletion for a number of highly oncogenic MMTVs, such as C3H- and GR-MMTVs, have been studied in great detail. Some work has also been done with the MMTVs expressed in two substrains of RIII mice, BR6 and RIIIS/J, but the nature of the interaction between T cells and the virus(es) that the parental RIII-strain of mice express has not been investigated. Since RIII mice (designated henceforth as RIII/Sa) have a very high incidence (90–98%) of mammary tumors, and they have been extensively used in studies of the biology of mammary tumor development, we have presently determined the pattern of Vβ-T cell deletion caused by RIII/Sa-MMTV-Sag(s) during viral infection. T cells were isolated from lymph nodes and thymus of young RIII/Sa mice, as well as from BALB/c (BALB/cfRIII/Sa), C57BL (C57BLfRIII/Sa), and RIIIS/J (RIIIS/JfRIII/Sa) mice after they were infected with RIII/Sa-MMTV(s) by foster nursing. The composition of the T cells was analyzed by FACS using a panel of monoclonal antibodies specific to a variety of Vβs. Our results show that milk-borne RIII/Sa-MMTV(s) infection leads to the deletion of $CD4^+$ Vβ-2, and to a lesser extent Vβ-8 bearing peripheral and central T cells in RIII/Sa, RIIIS/J, BALB/c, and C57BL mice. Our results are in contrast to the findings that C3H-, GR-, and BR6-MMTVs delete Vβ-14- and/or Vβ-15-specific T cells.

Keywords: RIII/Sa mouse; Mouse mammary tumor virus; Vβ genes; Clonal T cell deletion

Introduction

Mouse mammary tumor virus (MMTV) is the etiologic agent of mammary adenocarcinomas in mice (Nandi and McGrath, 1973; Moore et al., 1979). In most strains of mice, tumorigenic MMTVs transmit exogenously through milk, but in a few mouse strains the virus particles can be transmitted endogenously via the germ line. A unique feature of MMTV is that it exploits the immune system of the host for pathogenesis. The involvement of the immune system in MMTV-induced mouse mammary tumorigenesis was suspected as early as 1964 when neonatal thymectomy was found to reduce the incidence of mammary tumor development (Martinez, 1964). Subsequently, T cells were shown to play a role in MMTV infection of mammary cells in vivo (Tsubura et al., 1988). It is now well established that the antigen (superantigen, Sag) that interacts with the immune system in MMTV-infected mice is a product of the long terminal repeat (LTR) of the virus (Choi et al., 1991). The existence of this product was recognized initially by sequence analysis of the LTR and by in vitro translation studies of the viral RNA (Donehower et al., 1981; Dickson and Peters, 1981; Sen et al., 1981). Sag is a type II transmembrane protein that contains a small NH_2-terminal intracellular domain and a large extracellular COOH-terminus that interacts with the Vβ portion of the T cell receptor (Korman et al., 1992; Yazdanbakhsh et al., 1993; McMahon

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and Bogatzki, 1997). Sequence comparisons of the Sag proteins from some 35 endogenous and exogenous MMTV strains have shown striking similarities at the N-terminals, but there are two polymorphic regions in the C-terminal amino acids, 164 to 198, and from 288 to the C-terminus (Brandt-Carlson et al., 1993; Wrona et al., 1998). Variability in the amino acid sequence of this region among different MMTVs seems to be the determinant for contacts with different Vβ chains of the T cell receptor (TCR), for the induction of specific Vβ T cell proliferation when they are recognized as foreign, or for the deletion, during shaping of the immune repertoire, of the same subset of lymphocytes when they are present as products of endogenous MMTVs (for review see Held et al., 1994; Ross, 1998).

The Sags of endogenous and exogenous MMTVs have been grouped into several distinct families according to their Vβ specificity. This specificity correlates strongly with the observations that certain Sag molecules react with particular TCRs. The Sags are highly homologous within a given family but polymorphic between different families. MMTV Sags also differ in their abilities to induce a vigorous or a weak T cell response in a particular host. It is now well established that most, if not all, endogenous MMTVs, located on different chromosomes and heterogeneously distributed from one strain of mice to another, express their *Sag* genes irrespective of whether or not the transcripts of these proviruses are packaged into virions, and play some role in mammary tumor development. While the role of the Sags of exogenous viruses has been attributed to the survival of the virus through T cell-dependent expansion of infected B cells, the consequence of the expression of the Sags of endogenous MMTVs and the resultant deletion of specific Vβ T cells has been shown to provide the mice some protection from infection by exogenous viruses with the same Sag specificity (Golovkina et al., 1992). In addition, the *Sag*s of some nonpathogenic endogenous MMTV proviruses in certain strains of mice seem to contribute to viral propagation and tumor induction by copackaging with pathogenic exogenous or endogenous MMTVs (Golvkina et al., 1994, 1996, 1997). Examples exist that show that viruses with similar Sag specificity do not necessarily share similar biological activity. For example, the Japanese laboratory mouse strains CS and NC carry two unique endogenous MMTVs, *Mtv*-48 and *Mtv*-51, encoding similar T cell deletion ligands for Vβ-2, but only one of these proviruses (*Mtv*-48) produces milk-borne MMTVs (Niimi et al., 1995).

The endogenous proviruses *Mtv*-7, *Mtv*-43, and *Mtv*-50 as well as the exogenous MMTVs, JYG and SW, share the same Vβ-6, -7, -8.1, and -9 specificity, but these viruses differ significantly in their biological properties (McMahon and Bogatzki, 1997; Held et al., 1992; Nishio et al., 1994). For example, while both MMTV-SW and MMTV-JYG induce vigorous Sag-dependent T cell response in vivo after injection into native BALB/c mice, the MMTV-SW, unlike the MMTV-JYG, fails to induce mammary tumors (Held et al., 1992; Sarkar et al., 1994). The MMTVs, such as C3H and GR, that produce mammary tumors at a very high incidence in their natural hosts and/or in other strains of mice infected with these viruses have been shown to delete Vβ-14 and/or Vβ-15 (Marrack et al., 1991; Acha-Orbea et al., 1991). Footpad injection of MMTVs obtained from the milk of RIIIS/J mice, a derivative of cross-breeding between RIII and SEC/1ReJ mice, has also been shown to stimulate the proliferation of Vβ-14 T cells (Wajjwalku et al., 1995). This study neither addressed the question of whether RIIIS/J-MMTV Sag causes central and/or peripheral deletion of Vβ-14 T cells nor analyzed the structure of the Sag. Similarly, the structure of the Sag of a milk-borne MMTV carried by a substrain of RIII mice (BR6), derived from a crossing between an RIII male and a C57BL female mouse (Foulds, 1949), has been determined, but its Vβ T cell specificity has not been ascertained. The pathobiology of the MMTVs that the parental RIII mice express in their milk and/or in the tumors that they develop has been studied more extensively than the milk-borne viruses of BR6 and RIIIS/J mice (Moore et al., 1979). Surprisingly, no work has been done to determine how RIII-MMTV(s) infection modulates the Vβ T cell repertoire in susceptible hosts. Here, we demonstrate that the MMTV(s) that parental RIII mice (designated hence forth as RIII/Sa) express in their milk, unlike those viruses produced by the RIIIS/J and BR6 mice, delete T cells carrying Vβ-2- and Vβ-8-specific TCRs during infection.

Materials and methods

Mice

Four strains of mice, RIII/Sa, RIIIS/J, BALB/c, and C57BL, were used in this study. The progeny of a mammary tumor-bearing RIII female mouse on her fourth parity were isolated in 1974 and maintained as a separate lineage, designated RIII/Sa, in the laboratory of one of us (N.H.S.) at the Memorial Sloan-Kettering Cancer Center (MSKCC), New York. MSKCC obtained RIII mice from the laboratory of Dan Moore of the Institute of Medical Research, Camden, NJ. Records show that Dr. Moore received his RIII mice in 1950 from the laboratory of Dobrovolskia-Zavadskaia (Hilkens et al., 1981) and that he maintained the mouse colony by brother–sister mating (Moore et al., 1979). The RIII/Sa mice used in this study have also been maintained since 1974 by brother–sister mating of young adults from high-parity mothers (third or more). These mice develop mammary tumors at a very high incidence (>90%). RIIIS/J, BALB/c, and C57BL mice were obtained from Jackson Laboratory and were raised at the Medical College of Georgia. Although no recent data are available, RIIIS/J mice were known to carry both endogenous and exogenous MMTVs and to have a high incidence of mammary tumors (Schlom et al., 1973; Wajjwalku et al., 1995). BALB/c and

C57BL mice do not carry any exogenous MMTVs and rarely develop mammary tumors.

Virus infection via foster nursing and evaluation of TCR Vβ T cell deletion by antibody staining and flow cytometry

To determine the pattern of Vβ T cell deletion in RIII/Sa mice, as well as in other strains of mice, such as BALB/c, RIIIS/J, and C57BL infected with RIII/Sa-MMTV, lymphocytes obtained from these mice were analyzed. The latter groups of mice were infected by foster nursing of their newborn pups to RIII/Sa mothers. Lymphocytes obtained from the thymus of RIII/Sa mice were also analyzed to ascertain if MMTV infection resulted in central T cell deletion. T cells were analyzed for the expression of various mouse Vβs with the following monoclonal antibodies: anti-Vβ-2 (B20.6), -Vβ-3 (KJ25a), -Vβ-4 (KT-4-10), -Vβ-5 (MR9-4), -Vβ-6 (RR4-7), -Vβ-7, -Vβ-8.1/Vβ-8.2 (KJ16), -Vβ-8.2 (F23.2), -Vβ-8x (F23.1), -Vβ-9 (MR10-2), -Vβ-10 (KT10b.2), -Vβ-11 (RR3-15), -Vβ-12, -Vβ-13, Vβ-14 (14-2), and anti-TCR, H57-597. Most of the anti-Vβ antibodies used were from our own laboratory (L.I.); the antibodies raised against Vβ-7, -Vβ-12, and -Vβ-13 were purchased from PharMingen. Lymph node derived T cells were stained with each one of the abovementioned FITC-conjugated Vβ-specific MAbs and PE-conjugated anti-CD4 (GK1.5) antibody, as previously described (Ignatowicz et al., 1992; Scherer et al., 1995; Xu et al., 1996). At least 15,000 cells were analyzed on a FACScalibur (Becton–Dickinson, Mountain View, CA). The cells from the thymus were stained with anti-CD8 antibodies, in addition to the anti-CD4-, -Vβ-2-, Vβ-8x-, Vβ-8.1-.2-, Vβ-8.2-, and Vβ-14-specific antibodies. The percentage of Vβ-specific T cells was calculated from the total number of CD4-positive cells.

Virus infection by footpad injection

Milk was aspirated from third-parity RIII/Sa mice with the help of a mechanical pump, and virus was semipurified by centrifugation as described previously (Sarkar and Moore, 1970). One hundred microliters of a fivefold viral concentrate was injected into the four fatpads (25 μl/pad) of 8-10-week-old MMTV (exogenous)-negative BALB/c mice. Four days after virus administration, mice were killed; draining lymph nodes were harvested, and the cells were analyzed by flow cytometry.

Results

TCR-Vβ expression in RIII/Sa and RIIIS/J mice

In view of the fact that RIII/Sa and RIIIS/J mice were derived from a high mammary tumor incidence mouse strain, RIII, we assumed that both RIII/Sa and RIIIS/J carry the same strain of MMTV(s) and thus virus infection should affect the expression of TCR-Vβs in a similar manner. Preliminary analyses of splenic CD4-positive T cells from 4- to 6-week-old female mice showed significant differences in their distribution of Vβ-2, Vβ-3, Vβ-4, Vβ-5, Vβ-6, Vβ-8, Vβ-11, Vβ-12, Vβ-13, and Vβ-14 T cells (Fig. 1). Compared to RIII/Sa mice, RIIIS/J mice exhibited the presence of a much higher percentage of CD4-positive T cells carrying Vβ-2, Vβ-4, and Vβ-14. A reversal of this pattern of expression was found with Vβ-3, Vβ-5, Vβ-6, Vβ-8, Vβ-11, Vβ-12, and Vβ-13. The pattern of distribution of the various Vβ-carrying CD4-positive T cells from 8- to 10-week-old mice was found to be primarily similar to the pattern exhibited by the T cells from 4- to 6-week-old mice (data not shown). However, there was some decrease in the percentage of Vβ-2- and Vβ-8-expressing T cells in 8- to 10-week-old RIII/Sa mice compared to 4- to 6-week-old mice. The difference thus observed between the patterns of Vβ expression among the RIII/Sa and RIIIS/J mice was

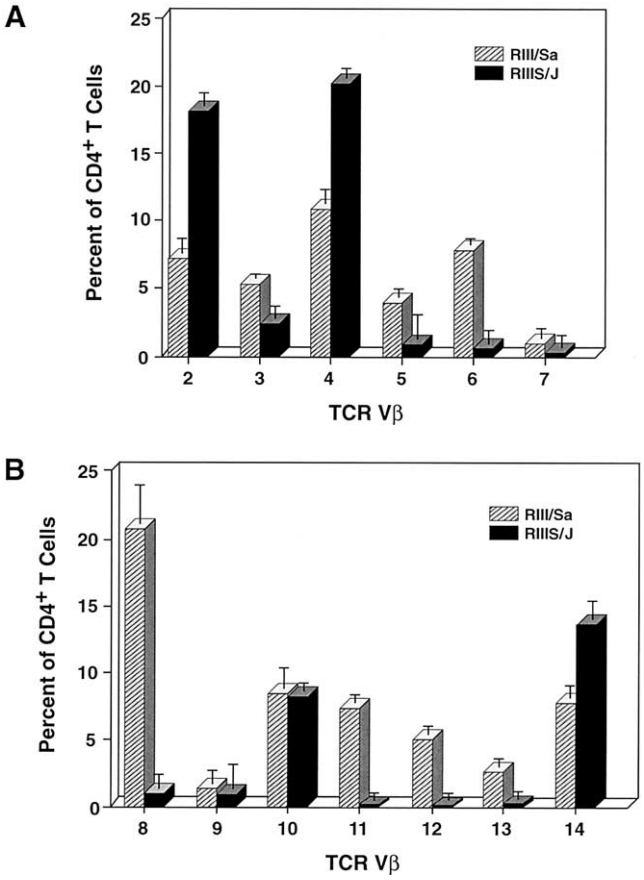

Fig. 1. TCR-Vβ repertoire of CD4-positive T cells in 4 to 6 week-old RIII/Sa and RIIIS/J mice. Cells from draining popliteal lymph nodes were harvested and stained with PE-labeled anti-CD4 and FITC-labeled anti-Vβ antibodies. Cells were then analyzed on a FACS CALIBUR flow cytometer. Each column represents the mean number of CD4-positive T cells and standard deviation of three to five mice. A: specificity for TCR Vβ-2, -3, -4, -5, -6, and -7; B: specificity for TCR Vβ-8, -9, -10, -11, -12, -13, and -14.

quite broad and we could not draw any conclusion as to whether or not the two mouse strains express the same or different endogenous and/or exogenous MMTV strains. The results probably reflected the diversity in the genetic background between the RIII/Sa and RIIIS/J mice.

Identification of the Vβs associated with RIII/Sa-MMTV infection

To analyze the effect of virus infection in mice free of milk-borne MMTV, newborn BALB/c mice, which have been shown to be susceptible to infection by a variety of MMTV strains, were foster-nursed by RIII/Sa females. At 6-8 weeks of age, the foster-nursed females were sacrificed, and cells from lymph nodes as well as T cell enriched splenocytes were harvested and analyzed by flow cytometry for a variety of TCR Vβ expression on CD4-positive T cells. Similar experiments were done with the splenocytes from BALB/c mice that were not exposed to RIII/Sa-MMTV. It was found that virus infection resulted in the deletion of Vβ-2-expressing T cells in BALB/c mice by more than 95% (Fig. 2A); Vβ-8-expressing T cells were also found to be deleted by approximately 56% (Fig. 2B). By contrast RIII/Sa mice foster-nursed on BALB/c mice did not show any deletion of either Vβ-2- nor Vβ-8-expressing T cells (data not shown). These observations indicated that the virus(es) that RIII/Sa mice shed in their milk interacted with Vβ-2- and Vβ-8-specific T cells.

It has been shown previously that foster-nursing as well as inoculation of milk samples or purified virus particles from the milk of parental RIII mice cause productive infection to C57BL mice (for review, see Moore et al., 1979). Therefore, we investigated whether or not RIII/Sa-MMTV infection would induce deletion of Vβ-2- and Vβ-8-specific T cells in this strain of mice. C57BL mice were foster-nursed on third-parity RIII/Sa mice. T cells were harvested from 6- to 8-week-old animals and analyzed using Vβ-2- and Vβ-8-specific antibodies; Vβ-14-specific antibody was used as a control. A second control experiment involved a group of BALB/c mice. These mice were foster-nursed on third-party RIII/Sa mothers that were the sisters of those mice used for foster-nursing the C57BL pups. This was done to minimize possible differences in the amounts of virus particles available for infection in the two groups of C57BL and BALB/c mice. As shown in Fig. 3A and B, RIII/Sa-MMTV deleted both Vβ-2- and Vβ-8-specific T cells in both mouse strains, but the extent of Vβ-2 T cell deletion appeared to be less dramatic in C57BL mice than what we observed in BALB/c mice. RIII/Sa-MMTV(s) also appeared to induce a lower level of Vβ-8-specific T cell deletion in C57BL mice than in BALB/c mice. Since the anti-Vβ-8 antibody used in these experiments contained also Vβ-8.1 and Vβ-8.2, we used two other TCR Vβ-8-related antibodies that were available to us: one contained both Vβ-8.1 and Vβ-8.2 specificity, while the other was a monoclonal antibody against TCR Vβ-8.2. As shown in Fig. 3A and B, RIII/Sa-MMTV infection resulted in Vβ-8.2, and possibly Vβ-8.1-specific T cell deletion in both BALB/c and C57BL mice. This observation suggests that both A^d/A^s and E^d molecules can present the RIII/Sa-MMTV(s) Sag.

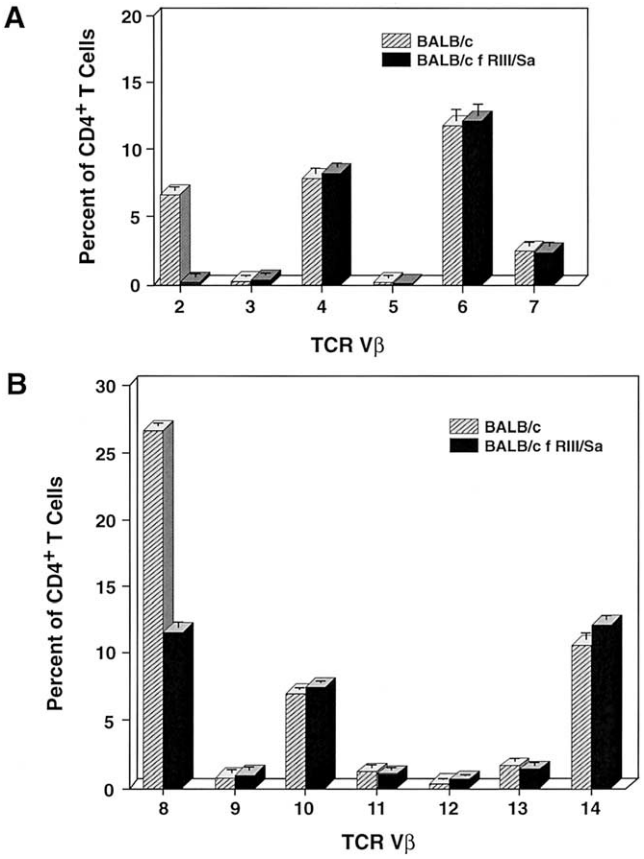

Fig. 2. The effect of milk-borne RIII/Sa-MMTV(s) infection on the TCR-Vβ repertoire in BALB/C mice. Newborn BALB/c pups were removed from their mothers and foster-nursed on RIII/Sa mothers. Popliteal lymph-node cells were harvested from 6-to-8-week-old foster-nursed mice, stained for CD4-positive T cells and for the expression of their Vβ gene products, and analyzed by flow cytometry. Same age group BALB/c mice nursed by their own mothers were used as controls. Note that significant deletion of T cells carrying only Vβ-2 ($P < 0.005$) and Vβ-8 ($P < 0.01$) specificity occurred in those BALB/c mice that were exposed to the milk of RIII/Sa mice. Data are expressed as mean and standard deviation (error bars) for three to five mice. A: specificity for TCR Vβ-2, -3, -4, -5, -6, and -7; B: specificity for TCR Vβ-8, -9, -10, -11, -12, -13, and -14.

As shown in Fig. 1, RIIIS/J mice carry a larger population of Vβ-2, Vβ-4, and Vβ-14 T cells compared to RIII/Sa mice, and since Vβ-2-specific T cells are one of the targets for the RIII/Sa-MMTV-Sag(s), we investigated whether RIIIS/J mice could be infected with RIII/Sa-MMTV(s). Deletion of Vβ-2-specific T cells was used as a marker for viral infection. RIIIS/J pups were foster-nursed by RIII/Sa mothers. Six to eight-week-old pups were sacrificed; lymph node T cells were prepared, stained with anti-Vβ-2, -Vβ-3, -Vβ-4, -Vβ-10, and Vβ-14 T cell specific antibodies, and analyzed by flow cytometry. The results showed that more than 90% of only Vβ-2-specific T cells were deleted in

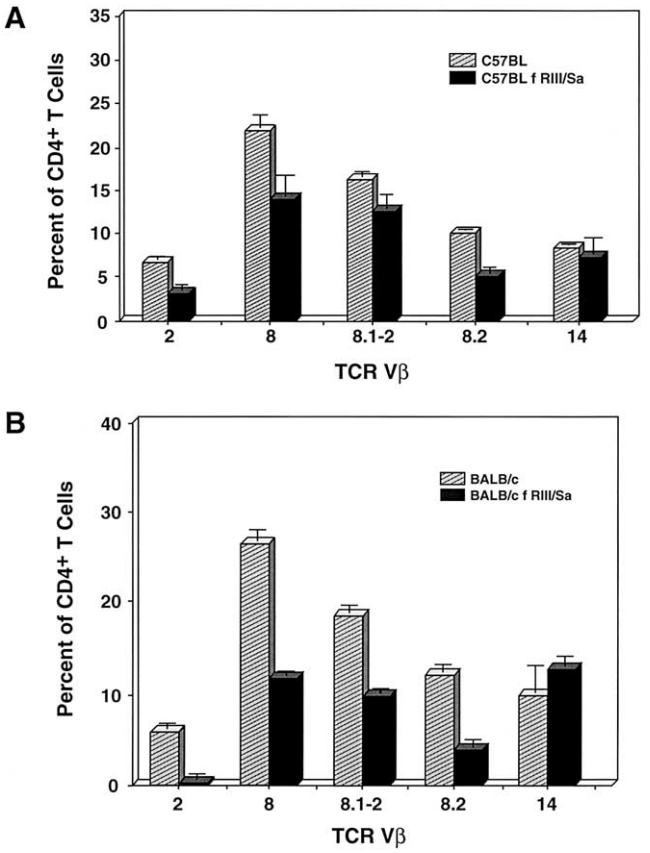

Fig. 3. Comparative studies of the levels of TCR Vβ-2- and Vβ-8-specific CD4-positive T cell deletions in C57BL (A) and BALB/c (B) mice exposed to RIII/Sa mouse milk. In this experiment, newborn pups from both C57BL and BALB/c mice were foster-nursed by a group of RIII/Sa mothers (sisters) of the same parity (third). C57BL and BALB/c pups nursed by their own mothers were used as controls. The experimental conditions and data analyses were the same as those described in Fig. 2 (also see text for details).

those groups of mice that were fed with RIII/Sa mouse milk (Fig. 4). This indicates that RIIIS/J mice are highly susceptible to RIII/Sa-MMTV(s).

Several investigators have shown that injection into adult mice of bacterial superantigen, MMTV-containing milk, or semipurified virus preparations results in an early increase in the proportion of T cells expressing the Vβ products that are specific for that superantigen. To evaluate further the specificity of T cell response in vivo to milk-borne MMTV(s) of RIII/Sa mice, exogenous MMTV-free adult BALB/c mice were injected with semipurified virus that was obtained from the milk of RIII/Sa mice or with PBS as controls. Four days later draining lymph nodes were harvested from both the experimental and the control groups. The lymph nodes from the experimental groups of mice were found to be two- to threefold enlarged compared to those mice that were injected with PBS. Flow cytometric analyses of the lymph node cells revealed that MMTV injection resulted in a dramatic increase in those T cells that were expressing TCR Vβ-2. The proportion of T cells expressing Vβ-8 was also found to be increased in the MMTV-infected mice. However, the relative increase in TCR Vβ-8 cells was lower than that exhibited by the TCR Vβ-2 cells. There was no increase in other TCR Vβ cells, including the TCR Vβ-14 cells (Fig. 5). These results may indicate that RIII/Sa mice express two different strains of infectious MMTVs, one of which interacts with Vβ-2 cells, while the other strain of virus interacts with Vβ-8 cells. Alternatively, these mice may carry only one strain of MMTV that interacts with both TCR Vβ-2 and Vβ-8 cells (see Discussion).

Time course of T cell deletion with RIII/Sa-MMTV infection

Different strains of MMTVs have been shown to produce different deletion patterns of TCR Vβ cells in the infected animals. Exogenous C3H- and GR-MMTVs appear to induce a slow deletion of T cells expressing Vβ-14 (Marrack et al., 1991; Acha-Orbea et al., 1991; Ignatowicz et al., 1992). By contrast, the time course of Vβ-2 cell deletion caused by BALB/cV-MMTV has been described to be significantly more rapid compared to both C3H- and GR-MMTVs (Ando et al., 1995). These observations prompted us to evaluate the deletion kinetics of both Vβ-2 and Vβ-8 cells in BALB/c and RIIIS/J mice after they were infected with milk-borne RIII/Sa-MMTVs by foster nursing. Our results show that, in general, RIII/Sa-MMTV(s) induces rapid deletion of Vβ-2 T cells not only in the parental RIII/Sa mouse strain, but also in BALB/c and RIIIS/J strains of mice (Fig. 6). It should be noted, however, that the kinetics of deletion in these latter strains of mice were found

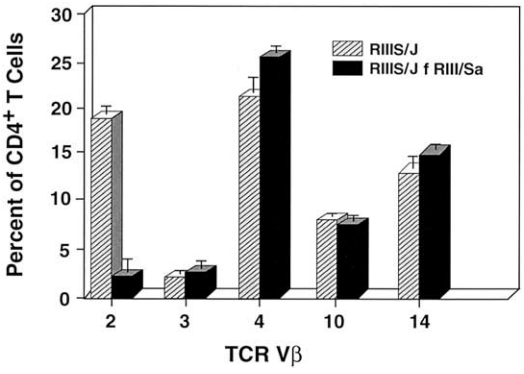

Fig. 4. Demonstration for the deletion of TCR Vβ-2 CD4-positive T cells in RIIIS/J mice infected with RIII/Sa-MMTV. Newborn RIIIS/J pups were foster-nursed by third-parity RIII/Sa mice and their lymph nodes were examined at the age of 6–8 weeks for the presence of CD4-positive T cells carrying TCR Vβ-2, -3, -4, -10, and -14. RIIIS/J pups nursed by their own mothers were used as controls. The experimental conditions and data analyses were the same as those described in Fig. 2 (also see text for details). Note that the lymph nodes of RIIIS/JfRIII/Sa mice contained approximately 85% fewer TCR Vβ-2 (CD4-positive) T cells than the lymph nodes of RIIIS/J mice (controls); the percentage of other TCR Vβ-T cells in both the control and the experimental groups were comparable.

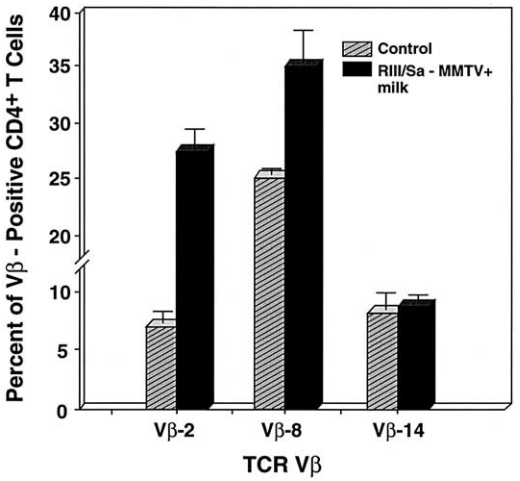

Fig. 5. Proliferative response of Vβ-2 and Vβ-8-specific CD4-positive T cells in adult BALB/c mice exposed to semipurified preparations of MMTV from the milk of third-parity RIII/Sa mice. Eight-to-ten-week-old BALB/c mice (free of milk-borne MMTV) were injected in the footpads with MMTV. Control mice were injected with PBS. Four days later, animals from both groups were sacrificed, and draining lymph nodes were harvested. Lymph node cells were analyzed by flow cytometry for the proportion of CD4-positive TCR Vβ-2 and Vβ-8 T cells. As control, the effect of virus infection on TCR Vβ-14 T cells was also analyzed. Data represent the mean number of cells and standard deviation for four individually analyzed mice. Note that TCR Vβ-2 T cells were expanded approximately by 3.6-fold ($P < 0.001$), whereas TCR Vβ-8 T cells were expanded by only a factor of 1.4-fold ($P < 0.01$).

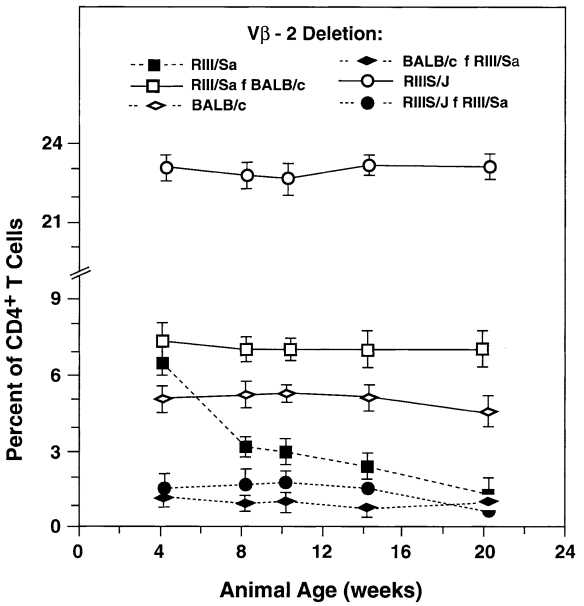

Fig. 6. Kinetics of TCR Vβ-2 T cell deletions in spontaneously MMTV(s)-infected RIII/Sa mice, and in experimental RIII/Sa-MMTV(s)-infected BALB/c (BALB/cfRIII/Sa) and RIIIS/J (RIIIS/JfRIII/Sa) mice. Exogenous MMTV-negative BALB/c and RIIIS/J mice were used as controls. Other control animals used were RIII/SafBALB/c; the removal of RIII/Sa-MMTV(s) in these mice was done by foster-nursing RIII/Sa pups to BALB/c mice. Data are expressed as the mean and standard deviation of four mice.

to be more rapid than in RIII/Sa mice. That the deletion of Vβ-2 T cells is specific to milk-borne RIII/Sa-MMTV(s) was further confirmed by analyzing the status of these cells in those RIII/Sa mice that were foster-nursed by BALB/c mice. The RIII/SafBALB/c mice did not show any evidence of Vβ-2 T cell deletion within the period of 20 weeks that we examined.

The time-course deletion of Vβ-8 cells in BALB/c mice infected with RIII/Sa-MMTV(s) showed a somewhat different pattern; the kinetics appeared to be slow (Fig. 7). This was also found to be true in RIII/Sa mice. It should be mentioned that we could not evaluate the kinetics of Vβ-8 T cell deletion in RIIIS/J mice because of the fact that the percentage of Vβ-8 cells in this strain of mice, as compared to RIII/Sa and BALB/c mice, is very low (see Figs. 1B and 2B). Taken together, it appears that RIII/Sa mice express in their milk a strain(s) of MMTV(s) that induces in the virus-infected host a rapid deletion of Vβ-2 T cells and a relatively slow deletion of Vβ-8 T cells.

RIII/Sa-MMTV infection induces deletion of central T cells

The results of our investigation into the deletion patterns of peripheral TCR Vβ T cells as shown above led us to address the question of whether or not RIII/Sa-MMTV infection also induces clonal deletion of Vβ-2 and/or Vβ-8-expressing immature thymocytes. T cells were prepared from thymus of 4-, 10-, and 16-week-old RIII/Sa mice that were raised by their own mothers producing milk-borne RIII/Sa MMTVs. $CD4^+$ and $CD8^+$ T cells were stained for

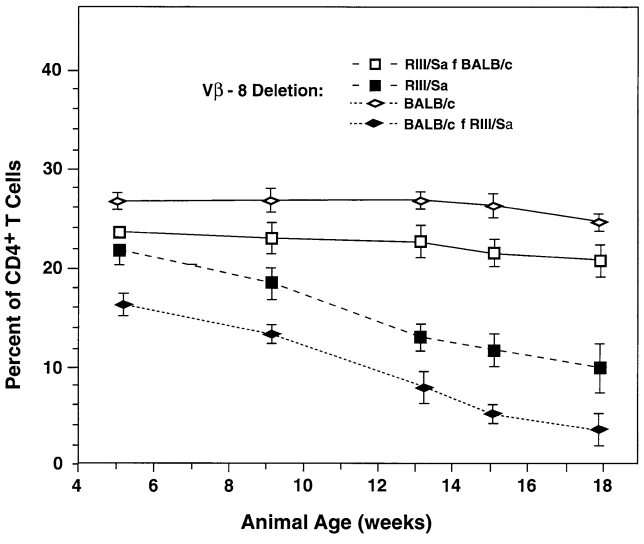

Fig. 7. Kinetics of TCR Vβ-8 T cell deletions in spontaneously MMTV(s)-infected RIII/Sa mice and in experimentally RIII/Sa-MMTV(s)-infected BALB/c (BALB/cfRIII/Sa) mice. Control animals included BALB/c and RIII/SafBALB/c mice. Data are expressed as the mean and standard deviation of four mice.

Table 1
Percentage of $CD4^+$ and $CD8^+$ T cells expressing Vβ-2, Vβ-8, Vβ-8.1/8.2, Vβ-8.2, and Vβ-14 in the thymus of RIII/Sa mice infected neonatally with milk-borne RIII/Sa-MMTV(s)

	TCR Vβ		
CD4/CD8	4-week-old mice	10-week-old mice	16-week-old mice
Vβ-2			
CD4	6.7 ± 1.2	5.0 ± 1.1	1.8 ± 0.3**
CD8	5.4 ± 0.8	4.1 ± 0.5	1.5 ± 0.2**
Vβ-8			
CD4	22.6 ± 1.0	21.6 ± 1.0	15.9 ± 0.5*
CD8	23.1 ± 1.4	24.2 ± 1.2	16.9 ± 0.3*
Vβ-8.1/8.2			
CD4	14.2 ± 0.9	12.9 ± 1.1	9.7 ± 0.5*
CD8	18.2 ± 1.0	18.9 ± 0.9	11.5 ± 0.4*
Vβ-8.2			
CD4	13.6 ± 0.9	10.4 ± 0.7	9.2 ± 0.6*
CD8	8.47 ± 0.7	13.2 ± 1.0	8.9 ± 0.6
Vβ-14			
CD4	4.2 ± 0.4	6.6 ± 0.6	6.3 ± 0.8
CD8	3.8 ± 0.3	4.1 ± 0.5	5.4 ± 0.5

Note. At the indicated ages, mice were killed and T cells were prepared from thymus and analyzed by two-parameter flow cytometry for TCR Vβ expression on $CD4^+$ and $CD8^+$ cells. Data represent the mean number of T cells from three individually analyzed mice. Deletion of $CD4^+$ Vβ-2 cells were significant by Student's *t*-test at $P < 0.005$ (**) in 16-week-old animals as compared to the 4-week-old mice.

* Significant at $P < 0.01$.

the expression of Vβ-2, Vβ-8, and Vβ-14. Flow cytometric analyses revealed that the thymus of virus-infected 16-week-old RIII/Sa mice contained significantly lower numbers of $CD4^+$ and $CD8^+$ TCR Vβ-2 cells than the thymus of 4-week-old mice (Table 1). The reactivity of the viral superantigen to $CD4^+$ cells appeared to be stronger than to the $CD8^+$ Vβ-2 T cells. This reduction of the T cells seems to be virus specific since, as expected, we did not see any changes in the proportion of TCR Vβ-14 cells in our virus-infected mice of different ages. TCR Vβ-14 cells have been shown to be deleted in mice infected with C3H-MMTV. Our results also show that T cells carrying Vβ-8, Vβ-8.1/8.2, or Vβ8.2 also suffer from deletion as a consequence of RIII/Sa-MMTV infection. However, the response of these particular subsets of T cells was much weaker than the TCR Vβ-2-expressing T cells. Overall, our data suggest that RIII/Sa-MMTV(s) infection induces deletion of not only peripheral mature T cells, but also of immature thymocytes.

Discussion

The initial events that facilitate MMTV infection of mouse mammary glands involve B cell infection followed by the expression of the viral superantigen, Sag, as a type II transmembrane glycoprotein at the B cell surface (Held et al., 1994). This allows the Sag-positive B cells to interact with $CD4^+$ T cells that express the appropriate Sag-reactive TCR Vβ domain. The outcome of such an interaction is the release of cytokines by T cells, enhanced proliferation of Sag-presenting B cells which facilitates the amplification of viral infection, and subsequent deletion of cognate T cells. In the present study, we have shown that the viruses (RIII/Sa-MMTVs) that RIII/Sa mice shed in their milk induce in the offspring the deletion of $CD4^+$ T cells expressing Vβ-2 and Vβ-8.1–8.3. Furthermore, experimental transmission of the virus in BALB/c, C57BL, and RIIIS/J, another strain of RIII-derived mice, also leads to the deletion of these two subsets of T cells. This finding is surprising because another virus, BR6-MMTV (often refered to as RIII-MMTV), which is shed in the milk of BR6 mice (a line derived from RIII mice) has been reported to delete Vβ-14 T cells (Xu et al., 1996). Similarly, one study has suggested the involvement of Vβ-14 T cells in RIIIS/J mice, a third subline of RIII mice that is thought to be infected with a milk-borne RIII-MMTV (RIIIS/J-MMTV) (Wajjwalku et al., 1995). In addition, although RIII-, C3H-, and GR-MMTVs have long been considered to be standard MMTV strains (Nandi and McGrath, 1973; Moore et al., 1979), unlike RIII/Sa-MMTV, both C3H- and GR-MMTVs have also been shown to induce the deletion of T cells expressing Vβ-14 (Xu et al., 1996; Ignatowicz et al. 1992). Two other strains of MMTVs, BALB14 and II-TES14 expressed in BALB/c and II-TES mice, respectively, have also been shown to bear Vβ-14 specificity (Golovkina et al., 1997; Ando et al., 1995).

A survey of the experimental results obtained by a large panel of investigators on the deletion patterns of the Vβ T cell repertoire as a consequence of the expression of various exogenous and endogenous MMTV-Sags shows relationships between the interaction of a particular population(s) of Vβ T cells with the Sags of an MMTV(s) having specific C-terminal amino acid sequences (Held et al., 1993; Frankel et al., 1991; Dyson et al., 1991; Woodland et al., 1991; Marrack et al., 1991). This type of finding allows one to suspect what MMTV strain is expressed in an unknown MMTV model in which the deletion of specific Vβ T cells has been experimentally determined, and vice versa. On the basis of this assumption, it could be hypothesized that our RIII/Sa mice most likely express two different strains of MMTVs belonging to two different virus groups with Vβ-2 and Vβ-8 specificities (see Fig. 8). We have tentatively designated the Vβ-2-specific RIII-virus as RIII/Sa-MMTV-I and the Vβ-8-specific virus as RIII/Sa-MMTV-2. It should be mentioned that we have cloned two different species of *Sag*-cDNAs by RT-PCR amplification of milk-borne viral RNA and determined their sequences (data not shown; see GenBank Accession Nos. AF136898 and AF136899). A comparison of the C-terminal 30 amino acids of the *Sag*s of these virions with those from other Vβ-2- and Vβ-8-specific MMTVs (Fig. 8) indeed indicate that at least two different strains of MMTVs are expressed in the milk of RIII/Sa mice.

The RIII/Sa-MMTV-1 strain corresponds to a group of both endogenous and exogenous viruses that include

Vβ Specifity	MMTV Strain	C-Terminal Sequences		
2	RIII/Sa-MMTV-1	KSR.EVQKH.	.DSIK---.L	PLSY*
2	BALB2	KSR.EVQKH.	.DSIK---.L	PLSY*
2	C4	KSR.EVQKH.	.DSIK---.L	PLSY*
2	*Mtv*-DDO	NSR.EVKKH.	..SIK---.L	PLSY*
2	II-TES2	KSR.EVQRH.	M.SIK---.L	PLSY*
2	CS/*Mtv*-48	KSR.EVQRH.	M.SIK---.L	PLSY*
2	BALB/cV	KSR.EVQRH.	.DSIK---.L	PLSY*
?	C3H-K	KSR.EVQRH.	.DSIK---.L	PLSY*
3,5,17a2	*Mtv*-6	NSR.EAKRHI	...IK---.L	PLSY*
8.1-8.3	Sa-MMTV/2	Y.E..AIAKI	LHNKKHTF.G	KL..DGLHFT*
2,6,8.1-8.3,14	FM	Y.E..AIAKI	LHNIKYTF.G	KL..DGLHFA*
7,8.1-8.3	SHN	Y.E..AIAKI	LHNKKTHF.G	KL..DGLHFA*
16	*Mtv*-RCS	Y.E..AIAKI	LHNKKHTF.G	KL..DKLHFT*
14	BR6	-TKERTVAGL	IEHYS---AK	TYGMSYYD*

Fig. 8. Identity RIII/Sa-MMTV-1 and RIII/Sa-MMTV-2, expressed in the milk of RIII/Sa mice, with other MMTV strains with TCR Vβ-2 or Vβ-8 T cell specificity and/or nearly identical C-terminal amino acids of the MMTV-Sag molecules. That RIII/Sa-MMTV-1 and RIII/Sa-MMTV-2 carry the specificity for the deletion of TCR Vβ-2 and Vβ-8 T cells, respectively, were experimentally determined in the present study. The C-terminal amino acid sequences of these two MMTV strains were derived from GenBank (Accession Nos. AF136898 and 136899). Information for Vβ-specificity and alignment of the last 30 amino acids of the Sags of other MMTVs were obtained from published reports (for example, see Wrona et al., 1998). Note that neither of the two RIII/Sa-MMTV strains show similarity with BR6-MMTV. Gaps (shown as dashes) were introduced to maximize amino acid identities and the dots indicate identical amino acids. The astericks indicate the position of the stop codons.

BALB/cV, BALB2, C3H-K, C4, CS/*Mtv*-48, *Mtv*-DDO, and II-TES2. These viruses are expressed in a variety of mouse strains and interact with Vβ-2 T cells (Wrona et al., 1998). Interestingly, the II-TES2 MMTV-expressing TES2 mice that were derived by cross-breeding between DBA/2 and Japanese pet mice (Wajjwulku et al., 1995) have been shown to express another viral strain, MMTV-II-TES14 (Ando et al., 1995). Similarly, the BALB2-MMTV-expressing BALB/cT (subline 7) mice, maintained at the National Institutes of Health, Frederick Cancer Research Facility, express another MMTV strain, MMTV-BALB14 (Golovkina et al., 1997). In terms of viral expression our RIII/Sa mice thus resemble TES2 and BALB/cT mice. However, while the Sag of MMTV-II-TES2 and MMTV-BALB2, similar to RIII/Sa-MMTV-1, interacts with Vβ-2 T cells, the other viral strains (MMTV-II-TES 14 and MMTV-BALB 14), unlike RIII/Sa-MMTV-2, target the Vβ-14 T cells. It should be pointed out that the similarity of the C-terminal sequences between different MMTV strains does not necessarily reflect their pattern of viral expression, the nature of the Sag interaction with Vβ-T cells, and the biological consequences of viral infection. For example, *Mtv*-48 and *Mtv*-51, which have been shown to be present in Japanese mouse strains CS and NC, respectively, are completely identical in their *orf* sequences, but unlike *Mtv*-48, *Mtv*-51 is incapable of producing a milk-borne virus (Niimi et al., 1995). The C3H-K virus was identified in a colony of BALB/c mice that had been foster nursed on C3H-MMTV-positive mothers (Wellinger et al., 1986). The C-terminal sequences of this virus are nearly identical to BALB/cV-MMTV, but the virus, although expressed in mammary glands, does not induce mammary tumors (Rollini et al., 1992).

Many of the endogenous and exogenous MMTVs have been shown to require major MHC class II-I-E expression for clonal deletion and stimulation. The classic examples for such a requirement is exhibited by two standard exogenous MMTV strains, the C3H- and GR-MMTVs (Ross, 1998). However, other exogenous viruses, such as BALB/cV and MMTV-C4, as well as the endogenous MMTV-DDO, do not depend on I-E expression for clonal deletion and stimulation (Shakhov et al., 1993; Kang et al., 1993; Jouvin-Marche et al., 1993; Tomonari et al., 1993; Hodes et al., 1993). It has been suggested that strong superantigens do not depend on MHC Class II I-E expression for clonal deletion and stimulation, and that the kinetics of clonal deletion may be correlated with the strength of superantigenic activity (Held et al., 1994). For example, MMTV-DDO induces vigorous stimulation and fast kinetics of clonal deletion, whereas MMTV-CS induces vigorous stimulation but slow kinetics of clonal deletion, despite the fact that the C-terminal amino acid sequences of these two viruses are nearly identical (Jouvin-Marche et al., 1993). Our results suggest that RIII/Sa-MMTV-1 infect both BALB/c (H-2^d I-E^+) and C57BL (H-2^b I-E^-) mice, implying that the Sag of this virus has a broader superantigenic specificity than the other members of this group of MMTVs, such as MMTV-II-TES2, the SAg of which requires MHC class II I-E molecules exclusively for antigen presentation (Ando et al., 1995).

As shown in the present study, the second virus that RIII/Sa mice express in their milk interacts with Vβ-8-specific T cells. Deletion of a specific subset of T cells carrying Vβ-8.1 has been shown to be induced as a consequence of the Sag expression of a relatively large group of endogenous and exogenous MMTVs consisting of *Mtv*-7, SW, *Mtv*-50, *Mtv*-43, JYG, FM, SHN, as well as *Mtv*-44 (Ross, 1998). The majority of this virus group also deletes Vβ-6, -7, and -9 (*Mtv*-7, SW, *Mtv*-50, *Mtv*-43, JYG). An exceptionally polymorphic pattern of Vβ-T cell deletion is exhibited by FM-MMTV. Unlike the other viruses in this group, the Sag of this virus appears to interact with both

TCR Vβ-2 and Vβ-14 cells. Because of the fact that the C-terminal 30–40 amino acids of the Sags of different MMTV strains are variable and correlated strikingly with recognition of particular TCR Vβ chains, how it is possible that the Sag of FM-MMTV interacts with such a variety of TCR Vβ cells, particularly the TCR Vβ-2 and Vβ-14 cells? Our MMTV-2 virus seems to interact with only Vβ-8-specific T cells, despite the fact that our preliminary results show that the C-terminal amino acids sequences of this virus resemble very closely the Sag sequences of FM-MMTV (Yoshimoto et al., 1994, 1996; Upragarin et al., 1997). It is possible that FM mice may express multiple strains of MMTVs, each of which interact with distinct subsets of Vβ T cells. The following observations may support this idea. First, the C-terminal amino acid sequences of the Sag of FM-MMTV differ significantly from the Sag sequences of the two distinct groups of MMTVs (C3H, GR, BR6, II-TES14, and BALB14; and BALB2, C4, MMTV-DDO, II-TES2, CS/MTV-48, and BALB/cV) with exclusive recognition for the Vβ-2- and Vβ-14-specific T cells. Second, our present work has shown that RIII/Sa mice express at least two distinct strains of MMTVs. This is consistent with the observations that other mouse strains, such as II-TES and CS, have been shown to express more than one MMTV strain. Finally, as suggested previously, new MMTV strains may be generated in some mouse strains via recombination of endogenous and exogenous viruses. It is, therefore, most likely that many, if not all of the available mouse strains, may express multiple MMTV strains. This raises an important biological question for those mouse models in which multiple strains of viruses are expressed: does each viral strain contribute to mammary tumor development? We are currently addressing this question in our RIII/Sa mouse model.

Our finding that the viruses that RIII/Sa mice express in their milk interact with Vβ-2- and Vβ-8-specific T cells is unexpected because previous studies with two other sublines, BR6 and RIIIS/J, derived from the original RIII mouse strain, have shown that both mouse lines express only one viral strain, the Sag of which interacts only with Vβ-14-specific T cells in a manner similar to that exhibited by C3H- and GR-MMTVs. Indeed the Sag sequences of BR6-MMTV are similar to the Sag sequences of C3H- and GR-MMTVs (Ross, 1998). Transfer of GR-MMTV in the form of an endogenous Mtv-2 provirus from GR mice to wild mice, free of both endogenous and exogenous MMTVs, also results in the deletion of Vβ-14-specific T cells (Morris et al., 1986; Ferrick et al., 1992). Since the Sag sequences of the putative RIIIS/J-MMTV was not reported in the work that showed the effect of RIIIS/J mouse milk on the deletion of Vβ T cells (Wajjwalku, 1995), we do not know the nature of the virus that these mice were expressing at the time of the experiments. Surprisingly, however, we could not confirm that the presently available RIIIS/J mice do indeed express Vβ-14-specific MMTV in their milk. Our results show that Vβ-14-specific T cells are not deleted in RIIIS/J mice (data not shown), and thus they may not produce any GR- or C3H-like MMTV particles in their milk. This view is consistent with the fact that RIIIS/J mice have currently been described in the Jackson Laboratory catalog as nonproducers of mammary tumors. It should be emphasized further that RIIIS/J mice differ significantly from RIII/Sa mice in that they have the largest known deletion of the TCR Vβ genes: Vβ-8 and Vβ-5 gene subfamilies, along with Vβ-6, Vβ-9, Vβ-11, Vβ-12, Vβ-13, Vβ-15, and Vβ-17 (Haqqi et al., 1989).

Presently, we cannot offer a definitive explanation for the apparent structural and biological differences observed between the MMTVs produced by RIII/Sa, RIIIS/J, and B6 mice, all of which were derived from the same mouse strain, RIII. It is quite likely, however, that the breeding history of these mice may have contributed to this problem. The RIII/Sa mice are the direct decendents of the parental RIII strain and thus should carry the wild-type RIII-MMTV. The BR6 mice were derived by breeding a C57BL female mouse with an RIII male (Foulds, 1949). This should have prevented any milk-borne RIII virus from transmission to the resultant offsprings, and thus the BR6 virus may represent a rarely expressed MMTV that was in the milk of that particular C57BL mouse.

The way the RIIIS/J mice were derived involved a number of breeding steps. Around 1967, neither RIII/J nor RIII/AnJ mice (same as RIII, with a high incidence of mammary tumors) were able to produce viable young at The Jackson Laboratory. Thus the laboratory bred an RIII/AnJ female with a male SEC/1ReJ mice (unrelated to RIII, but related to C3H; known to produce milk-borne MMTV and having a high incidence of mammary tumors; Staats, 1976). An F1 female from this cross was bred to an RIII/J male mice (see JAX's website: Http://jaxmice.jax.org/jaxmice-cgi//jaxmicedb.cgi?objtype=pricedetail&stock). Inbreeding of the resultant pups continued. This substrain was initially called RIII/2J, but is presently known as RIIIS/J (Sweeney et al., 1990). The breeding protocol might have contributed to the deletion of a large number of TCR Vβ genes in this strain (Haqqi et al., 1989). It was expected that RIIIS/J mice would retain the milk-borne RIII-MMTV, and indeed RIIIS/J mice were described to produce MMTV (Schlom et al., 1973; Wajjwalku et al., 1995). However, since the TCR Vβ-specificity of RIIIS/J virus has been shown to be similar to C3H-, GR-, and BR6-MMTVs (Wajjwalku et al., 1995), it might have evolved through recombination between a putative endogenous MMTV and an exogenous RIII virus.

As shown in the present study, the RIIIS/J mice, however, are highly susceptible to RIII/Sa-MMTV-2 virus infection which results in rapid deletion of TCR Vβ-2 T cells. Thus the RIIIS/J mouse model offers a unique opportunity for the isolation of RIII/Sa-MMTV-2 that is expressed together with RIII/Sa-MMTV-1 in the milk of RIII/Sa mice. Pups of RIIIS/J mice could be foster-nursed by RIII/Sa mice, or young RIIIS/J animals could be injected with milk

samples collected from RIII/Sa mice. RIIIS/J mice thus exposed to the milk of RIII/Sa mice are expected to be infected by RIII/Sa-MMTV-2, but not by RIII/Sa-MMTV-1, because these mice lack TCR Vβ-8 T cells, the target cells that are needed for the establishment of infection by RIII/Sa-MMTV-1.

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